PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/51, C07K 14/18, G01N 33/576

A1

(11) International Publication Number: WO 97/39129

(43) International Publication Date: 23 October 1997 (23.10.97)

(21) International Application Number: PCT/IT96/00248

(22) International Filing Date: 9 December 1996 (09.12.96)

(30) Priority Data:
RM96A000245 17 April 1996 (17.04.96) IT

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: NON-A-NON-E HEPATITIS VIRUS HAVING A TRANSLATABLE CORE REGION, REAGENTS AND METHODS FOR THEIR USE

(57) Abstract

It is described a non-A-non-E hepatitis virus (GBV hepatitis virus) having a translatable functional core region, polynucleotides, peptides, antibodies, diagnostics and reagents thereof and methods for their use.

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NON-A-NON-E HEPATITIS VIRUS HAVING A TRANSLATABLE CORE REGION, REAGENTS AND METHODS FOR THEIR USE

The invention concerns nonA-nonE hepatitis virus (GBV hepatitis virus) having a translatable 5' core region, polynucleotides, peptides, antibodies, diagnostics and reagents thereof and methods for their use.

Simons J.N. et al. (Nature Medicine, 1, 564, 1995) revealed a viral genome, Flavivirus-like, named GBV-C, in a small percentage of nonA-nonE hepatitis affected patient sera. The genome has some homology with genomes from two virus, GBV-A e GBV-B, identified in tamarins (Simons J.N. et al., Proc. Natl. Acad. Sci. USA, 92, 3401, 1995). Said virus represent a phylogenetic separate group, not comprising hepatitis C virus (HCV).

PCT patent application N. WO95/21922, in the name of Abbott Lab., shows a partial nucleotide sequence of a GBV-C isolate. A method to reveal antibodies against GBV is claimed (claim 44, p. 618); however no enabling disclosure for such method able to detect with a statistic significative percentage positive samples from nonA-nonE patients is provided. PCT patent application N. WO95/21922 does not disclose aminoacid sequences to be effectively used to design diagnostic peptides. As a matter of fact, Figure 39 shows a panel of proteins, having an around 300 aminoacid minimal lenght. Only 2 out 512 sera from drug-addicted subjects result to be positive for these proteins in an ELISA assay. Moreover at p. 168, table 23, ELISA assays on nonA-nonE patients result to be positive in very few samples, thus rendering the assay not workable: 0/89 Japan subjects; 0/67 Greece subjects; 6/72 USA (set M) subjects; 1/64 USA (set T) subjects; 3/62 USA (set 1/3) subjects; 18/32 Egypt subjects. At p. 169, table 24, only 21 out 303 nonA-nonE patients are positive with an ELISA assay using peptides

sequence. It is evident that said assay may not be used neither for a screening of healthy subjects, nor for diagnosis.

PCT patent application N. WO95/32291, in the name of Genelabs, identifies by epitope mapping different antigenic regions of the GBV polyprotein. However no peptides or a combination thereof for a diagnostic assay are disclosed.

EP patent application N. 736601, in the name of Abbott Lab., discloses 88 nucleotides upstream of the sequence shown in the PCT patent application N. WO95/21922. However no evidence is provided for an open reading frame in this region able to code for a core-like protein.

The nucleocapsid/E1 junction of the viral isolate was identified by Leary, T. P., et al. 1996. J. Med. Virol. 48:60-67. It is predicted to occur approximately 46 aminoacids downstream of the first methionine residue of the ORF, while Linnen, J. et al. 1996. Science 271:505-508 have also identified an isolate in which the putative capsid protein contains 83 aminoacid residues. The apparent main difference among these two isolates is one base deletion causing different initiation sites. Thus, in comparison with other *Flaviviridae*, in which the lenght of the capsid protein varies from 114 aminoacids in dengue fever virus to 175 residues in HCV, the GBV-C nucleocapsid appears too short to be functional.

The presence of core-like proteins in GBV-C has also been challenged by recent translation studies demonstrating that the site of translation initiation of the viral polyprotein is immediately upstream of the putative signal sequence for the E1 glycoprotein (Simons, J. N., et al. 1996. J. Virol. 70:6126-6135).

Although it was originally suggested that the apparent lack of a functional core protein could be ascribed to artefacts of the sequencing process, due to

compressions caused by the high GC contents (Leary et al. supra), it is now clear that these sequences represent the true status of GBV-C in the numerous isolates that have been characterized (Simons et al. 1996, supra).

It has been proposed, therefore, that the NH_2-terminal end of the GBV-C polyprotein is a truncated derivative of an ancestral capsid protein and that GBV-C requires a helper virus to replicate (Heringlake, S., H. L. Tillmann, and M. P. Manns. 1996. J. Hepatol. 25:239-247). Alternatively, since a limited number of virus clones from each isolate has been examined so far, it is possible that, after infection, a large population of single base deletion mutants arise and these mutants would have the highest probability of being examined.

The authors report the characterization of a GBV-C isolate, obtained from a HIV and HCV positive patient, in which translation can initiate at an AUG codon located 273 nucleotides upstream of the previously identified initiator site (Simons et al. 1996, supra), leading to a potentially functional nucleocapsid core protein of 107 aminoacids. This phenotype remained unvaried over the one year monitoring of the patient, suggesting therefore that stable replication competent GBV-C isolates may exist in nature.

The authors of the instant invention have also identified NS3 peptides of nonAnonE associated virus and has set up an assay to detect antibodies able to recognize epitopes comprised in said peptides. Said antibodies are detected in chronic and acute hepatitis affected subjects, classified as nonA-nonE for the lacking of known virus related markers, in a high percentage of studied subjects (around 30%). Surprisingly the assay is able to detect said antibodies also in subjects which are affected by other pathologies, as hepatitis A, hepatitis B, hepatitis C, hepatitis Delta,

HIV+, HIV+ "piastrinopenia", AIDS, autoimmune diseases (LES, ANA+), showing a relationship with GBV virus.

The assay is performed preferentially by reacting a treated biological sample, as serum, with selected peptides to get an effective and diagnostic method. Antigen-antibodies complexes are revealed in an effective and reproducible way by means of one of assays known to the expert in the field, as ELISA, RIA, EIA, immunoblotting, etc.

In the following "GBV virus" means virus associated to nonAnonE hepatitis; "immunologically homologous epitope" means an epitope having an aminoacid sequence which differs from the referring epitope for one or more aminoacids, but which is able to recognize and bind to antibodies for the referring epitope, with substantially the same binding affinity.

It is an object of the invention a peptide able to specifically bind to nonAnonE hepatitis virus antibodies, having a sequence of at least 10 aminoacids comprised either in SEQ ID No. 1:

RVRDVARGCGVQLVLYATATPPGSPMTQHPSIIETKLDVGEIPFYGHGIP
LERMRTGRHLVFCHSKAECERLAGQFSARGVNAIAYYRGKDSSIIKDGDL
VVCATDALSTGYTGNFDSVTDCGLVVEEVVEVTLDPTITISLRTVPASAE
LSMQRRGRTGRGRSGRYYYAGVGKAPAGVVRSGPVWSAVEAGVTWYGMEP
DLTAN;

or in SEQ ID No. 2:

MSLNRARYPPGLTTPTYGPRRPSMSLLTNRFNRRVDKDQWGPGVMGKDPK
PCPSRRTGKCMGPPSSAAACSRGSPRILRVRAGGISLPYTIMEALLFLLG
VEAGAIL;

or an immunologically homologous variant thereof.

It is a further object of the invention a peptide able to specifically bind to nonAnonE hepatitis virus antibodies, having a sequence of at least 10 aminoacids, said nonAnonE hepatitis virus being characterized by having a translatable functional core-like protein; or an immunologically homologous variant thereof.

Preferably said translatable functional core-like protein is longer than 84 aminoacid residues and comprises an aminoacid sequence at least 60% homologous to the sequence of SEQ ID No. 3: MSLLTNRFNRRVDKDQWGPGVMGKDPKPCPSRRTGKCMGPPSSAAACSRGSPRILRV RAGGISLPYTIMEALLFLLGVEAGAIL; or an immunologically homologous variant thereof.

More preferably said translatable core-like protein longer than 84 aminoacid residues further comprises an aminoacid sequence at least 60% homologous to the sequence of SEQ ID No. 4: MSLNRARYPPGLTTPTYGPRRPS, or an immunologically homologous variant thereof.

It is a further object of the invention a composition comprising at least one of the peptides of the invention. Preferably said composition is adhered on a solid phase.

According to a preferred embodiment of the invention said composition comprises at least two peptides of the invention.

Further object of the invention is a diagnostic kit for nonAnonE hepatitis comprising as specific reagent at least one peptide of the invention. Preferably said kit comprises at least two peptides of the invention.

Further object of the invention is a method to detect nonA nonE hepatitis virus antibodies in a sample comprising the following steps:

- incubating in conditions allowing the formation of an antigen-antibody complex said sample with at least one peptide of the invention;
- revealing said complex.

The invention is now described in a non-limiting way by reference to the following figures wherein:

- figure 1 represents the aminoacid sequence of GBV NS3 region and of used peptides, with their denomination;

- figure 2 represents a bar graphic of p3.1-p3.9 peptide reactivity in ELISA assay with sera from subjects affected by different pathologies;

- figure 3a e 3b represent aligned aminoacid sequences of GBV core region; first three from top: from literature, others: isolated from the authors; "-" means an identity with the U36380 sequence; "=" means an identity with the U45966 sequence;

- figure 4a represents aligned aminoacid sequences of a segment of GBV core region, from literature: the first from the top from U45966 of Fig. 3a, others are isolated from the authors; at the bottom the consensus sequence is shown (SEQ ID No. 3);

- figure 4b represents a bar graphic of consensus peptide of Fig. 4a reactivity in ELISA assay with sera from subjects affected by different pathologies;

- figure 5 represents the nucleotide sequence alignment of 10 GBV-C isolates obtained from 10 distinct GBV-C positive patients, compared with the sequences of the published isolates deposited in GenBank [GBV-C isolate (Leary et al., supra), PNF2161 isolate (Linnen et al. supra) named GBV1 in the figure, and R10291 isolate named GBV2 in the figure]. The sequences were analyzed with the CLUSTAL alignment program. The alignment includes the nucleotide sequence between AC1S and AS-CWP primers (shadowed in grey), and comprises a partial 5'UTR, the putative core region and a partial E1 region (corresponding to nt 112-733 of GBV-C, nt 129-752 of GBV1 and nt 56-680 of GBV2). The nucleotides are numbered only for isolate 35 (identified as the isolate with the longer ORF) and nucleotide +1 identifies the nucleotide A of its first putative start codon in frame with the long ORF. Note that in the other isolates nucleotide insertions or deletions are present. Gaps, introduced in the sequences to preserve alignment, are represented by dashes. The sequences between nucleotides 316 and 446, that are

conserved, are not shown for reason of space. The nucleotides conserved among all the sequences are pointed out by an asterisk. The ATG codons in frame with the long ORF of each isolate are boxed;

- figure 6 represents the aminoacid sequence alignment of the isolates described in Fig. 5 and deduced from their nucleotide sequences. The aminoacid sequence of isolate 35 is identified as 35/1, while the sequences deduced from the two additional isolates derived from distinct serum samples collected during the follow-up of this patient are pointed out as 35/2 and 35/3. The sequences were analyzed with the CLUSTAL alignment program. The sequence of the longer published ORF (GBV2) is considered as the consensus sequence. Dashes denote identity with the consensus sequence, whereas individual variations are shown by the single aminoacid code. The methionines in-frame with the long ORF are boxed and pointed out with an arrow for each isolate. The areas shadowed in grey mean the regions of high variability detected in our isolates;

- figure 7 represents a SDS-gel pattern of [^{35}S]cysteine-labelled in vitro translation products of the constructs, containing the AC1S/AS-CWP amplified region from the pointed out isolates. Positions of molecular weight standards (in kilodaltons) are pointed out. The last lane contains the negative control (no RNA). The IVTT of pCR35/AC1S construct was performed with and without capped mRNA;

- figure 8 represents the construction of the deletion mutants of pCR35/AC1S, pCR19/AC1S, pCR3064/AC1S and pCR2095/AC1S vectors. In the figure, the strategy for the construction of the pCR35/AC1S derivatives is described. The location of the T7 promoter (in black), the Bam H1 cleavage site and the position of the primers (AC1S and AS-CWP) used for the construction of the parent plasmid are shown. The numeration of the nucleotides is

relative to the first putative start codon of the sequence of isolate 35. The partial 5' non coding region is white, the partial ORF is shadowed in grey and the position of AUG codons are pointed out with the notation Met. The positions of the sense primers (primers 1, 2, 3, 4 and 5) used with the antisense AS-CWP primer for the construction of the deletion mutants are also shown. The same strategy was followed for obtaining the pCR19/AC1S, pCR3064/AC1S and pCR2095/AC1S derivatives. The length of the partial 5'UTR, of the partial ORF and the position of the AUG codons of these isolates are shown in Fig. 5 and 6.

- figure 9 represents a SDS-gel pattern of [^{35}S]cysteine-labelled in vitro translation products generated by the Δ2, Δ3, Δ4 and Δ5 deletion mutants of pCR35/AC1 (A), pCR19/AC1S (B) and pCR2095/AC1S (C) plasmids, obtained as described in Fig. 8. Positions of molecular weight markers (in kilodaltons) are pointed out. In panel (A) the products of capped and uncapped mRNAs derived from the pCR35/AC1S undeleted plasmid are shown. All the other mRNAs used in these experiments were capped transcripts. In the same panel the first lane represents the product of translation of an unrelated mRNA (UNR). In panel (B) the first lane represents the product of translation of the pCR35/λ mutant, that was used as control for the size of the product of isolate 19. In panel (C) the first lane contains the negative control (no RNA).

NS3 REGION

Analysis of GBV protein sequences

Protein sequences have been computer analyzed by means of ANTIGEN program 6.85 (PCGene). ANTIGEN utilizes Hopp and Wood algorithm to predict protein potentially hydrophilic regions.

Peptide synthesis

Peptides have been synthesized with an automated synthesizer MilliGen 9050 (Septrim). All of aminoacids were Fmoc α-amino protected and pre-activated at the carboxy terminus pentafluorophenyl esters; only serine and threonine were activated as benzotriazine esters.

All of coupling reactions were performed with HOBT 0.33M/DMF; washings were made with DMF and the α-amino group was released with 20% piperidine in DMF. Peptides were released with from resin by treatment with a mixture of TFA and scavengers which differ according to the composition.

Cases

sera from 52 healthy donors controlled to be HBV-, HCV- and HIV-;
sera from 27 acute nonAnonE hepatitis patients;
sera from 16 chronic nonAnonE hepatitis patients;
sera from 61 acute hepatitis B patients;
sera from 14 chronic hepatitis B patients;
sera from 15 acute hepatitis C patients;
sera from 15 chronic hepatitis C patients;
sera from 29 drug addicted HIV-, HCV+ patients;
sera from 31 HIV+ patients;
sera from 33 HIV+ piastrinopenic patients;
sera from 30 AIDS patients;
sera from 39 LES patients.

ELISA assay

The solid phase was prepared onto polivinylchloride dishes (Nunc), by using a peptide or a mixture thereof. Peptides were dissolved separately in a mixture of 50% CH_3CN/H_2O and diluted to a single final concentration of 10 µg/ml (total peptide concentration of 20 µg/ml) in carbonate buffer 50 mM, pH 9.6. 200 µl were added to each well and incubated for 2 hrs at 37°C. When a single peptide was used, the final concentration was 20 µg/ml.

Following to peptide adsorption, solid phases were saturated for 1 hr at 37°C with 300 µl/well of a BSA 2% solution in Tris-HCl 0.1M pH 7.5.

200 µl/well of 1:21 diluted sera in a solution of PBS containing 0.3 g/l EDTA, 2 g/l BSA, 2 ml/l Triton x100 and 2 ml/l Tween 20 were incubated 2 hrs at 37°C.

Following to 4 washes with PBS/Tween 20 0.1%, dishes were treated with 100 µl/well of horseradish peroxidase conjugated anti human Ig (Amersham), 1:30.000 diluted in a PBS solution containing 0.04% Tween 20 and 20% non immune goat serum.

Following to 4 washes with PBS/Tween 20 0.1%, 100 µl/well of chromogen/substrate were added (Sorin Biomedica Diagnostics, DEIA KIT). The colorimetric reaction was stopped after 30 min. by adding 200 µl/well of sulphuric acid 1N. The optical density was read at 450 nm by referring to 630 nm.

CORE REGION

Reverse transcription (RT)-PCR

Analysis was performed with serum samples from 10 distinct subjects resulted positive for the presence of GBV-C RNA with the RT-PCR and hybridization assay as previously described (Fiordalisi, G., et al. J. Infect. Dis. 174:181-183). Four of them (patients identified with the codes 35, 19, 47 and MG) were HIV+/HCV+ patients with chronic liver disease; DM and 1159 were non A-E acute hepatitis patients; BZ was a patient with hepatocellular carcinoma; 3064 was a patient with fulminant non A-E hepatitis; patient 2095 was an HCV+ subject with chronic hepatitis; and finally DS39 was a healthy blood donor. Total RNA was extracted from 100 µl of serum samples of these subjects with TriPure reagent (Boehringer Mannhein, Germany) and subjected to RT with random hexanucleotides using SuperScript II (Life Tecnologies, Gaithersbul-g, MD). The obtained cDNA was used as template for PCR reaction with degenerated primers, designed in our

laboratory and derived from the sequences of GBVC and GBV isolates deposited in Genbank database (Leary et al., Linnen et al. supra). The sequences of these primers, located in the 5'-UTR and putative structural regions, were:

AC1S sense primer:

5'AGG GTT SGW WGG TSG TAA ATC C 3' (SEQ ID No.5)

AS-CWP antisense primer:

5'CGC CTG RTA NAR NGG CCA RCA 3' (SEQ ID No.6)

wherein S = C or G; W = A or T; R = A or G; N = A or C or G or T.

Each of the 45 cycles of PCR included 1 min at 94°C, 1 min at 52°C and 1 min at 72°C, with an elongation time of 10 min in the last cycle. The sequence of the PCR product obtained with these primers corresponds to nt 129 to 752 of GenBank accession no. U44402, the GBV-C isolate with the longest core-like protein reported to date (Linnen et al. supra).

Cloning and sequencing

The amplification products obtained with AC1S and ASCWP primers were inserted into pCR2.1 vector (Invitrogen, San Diego, CA) and the sequences of the cloned inserts were obtained using the A.L.F. DNA sequencer (Pharmacia Biotech, Uppsala, Sweden). The sequenced plasmids were named pCR X/AC1S, where X corresponded to the isolate derived from the 10 patients considered in this study. Nucleotide and aminoacid sequences were analyzed with the CLUSTAL alignment program included in PC/GENE software package (Intelligenetics Inc., Mountain Vlow, CA).

Construction of deletion mutants

Mutants with deletions of different portions of the 5' region of pCR35/AC1S, pCR19/AC1S, pCR3064/AC1S and pCR2095/AC1S constructs were derived from these plasmids by PCR amplification using synthetic primers. The antisense primer was the AS-CWP primer described above,

while the sense primers, designed in our laboratory and derived from the sequences of our GBV-C isolates, mapped just upstream of the first 5 putative start codons on the basis of the sequence of isolate 35 (Fig. 5 and 6), as described in Fig. 8.

Primer 1: nt -43/-27

5'ACGTAAGCTT A GGT GTW GGY CCT ACC G 3' (SEQ ID. No.7)

Primer 2: nt +48/+64:

5'ACGTAAGCTT G TAC GGY CCA CGT CGC C 3' (SEQ ID. No.8);

Primer 3: nt +99/+117:

5'ACGTAAGCTT G CGA GTT GRC AAG GAC CAG 3'(SEQ ID. No.9);

Primer 4: nt +167/+183:

5'ACGTAAGCTT GR RVC GGG AAA TGC ATG 3'(SEQ ID. No.10);

Primer 5: nt +239/+255:

5'ACGTAACCTT TG AGG GCG GGT GGC ATT 3'(SEQ ID. No.11),

wherein underlined nucleotides correspond to specific sequences of GBV-C, W = A or T; Y = C or T; R = A or G; V = C or A or G. The position of the primers corresponded to the nucleotide numbers obtained considering as +1 the nucleotide A of the first ATG in the ORF of isolate 35 (see Fig. 5). The products of PCR with 1, 2, 3, 4 and 5 sense primers and AS-CWP antisense primer were cloned in pCR2.1 and named respectively pCRX/Δ1, pCRX/Δ2, pCRX/Δ3, pCRX/Δ4 and pCRX/Δ5, where X corresponded to the identification code of the isolate (35, 19, 3064 and 2095).

In vitro transcription and translation (IVTT)

Plasmids were linearized by digestion with BamH1 and transcribed in vitro with T7 RNA polymerase (mCAP mRNA Capping kit, Stratagene, La Jolla, CA) according to the manufacturer's instructions. In most cases, a m^7GpppG cap structure was incorporated at the 5' end of the RNA transcripts. In vitro translation reactions were performed with a rabbit reticulocyte lysate (RRL) system (Amersham, Buckinghamshire, England), according to the

manufacturer's instructions. Reactions (25 µl) contained 20 U of rRNasin (Promega, Madison,WI), 20 µCi of [^{35}S] cysteine (1.000 Ci/mmol, Amersham, England) and 1.5 µg of in vitro transcribed RNA. After incubation at 30°C for 1 hr, 2 µl aliquots were denatured for 5 min at 100°C in an equal volume of 2 X sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and analyzed on 18% SDS-polyacrylamide gels. Protein gels were dried and exposed to X-ray film.

RESULTS

NS3 REGION

The analysed peptides are comprised in the NS3 region, from aa. 1346 to aa. 1550 (SEQ ID No.1), and are shown in Figure 1.

All of peptides were adsorbed to the solid phase at different concentrations and confronted with a panel of sera from nonAnonE affected patients and healthy donors.

ELISA assays were performed with different concentrations and adsorption conditions. Results show that peptides of Table 1 react with sera with different binding affinities. The reactivity may be increased by previous adsorption to the solid phase of a mixture of different peptides.

The G20T peptide has a decreased activity given to a cysteine dimer at position 1467. The cysteine to serine (G20T(C->S)) substitution solves this stability problem.

In order to get good reactivities solid phases should be prepared either with a mixture of 10µg/ml S16V and 10 µg/ml G20T(C->S) or with E21A peptide at 20 µg/ml.

To determine the cut-off reactivities of 100 sera from healthy donors were tested and the following formula was applied:

OD cut-off = average OD healthy donors + 8 s.d. (standard deviation)

The value was for both of solid phases of 0.450 OD_{450nm}. All of samples with an OD>0.450 were considered

to be positive. Results from sera screening were reported in Table 1.

Table 1

			S16V+G20T(C-S)		E21A	
		n. patients	patients +	% +	patients +	% +
HEALTHY DONORS		52	0	0	0	0
HEP. nAnE	acute	27	8	32	7	28
	chronic	16	8	32	7	28
HEP.	B acute	61	30	46,9	17	26,6
	B chronic	14	1	3,1	0	0
	C acute	15	8	19,5	3	7,3
	C chronic	15	9	22,5	5	12,5
TD HIV-,HCV+		29	17	32,7	19	36,5
HIV+		31				
HIV+ piastrinopenic		33				
AIDS		30				
LES		39				

Results obtained with p3.1-p3.9 peptides of Figure 1 are shown in Figure 2.

Data show trhat some of analyzed peptides are able to specifically detect antibodies against epitopes of NS3 region of GBV virus.

CORE REGION

Identification of specific epitopes of "core" region

Aminoacid sequences (as in figure 3a e 3b) show methionines which may be aligned with either the first or the second methionine already described. The 35HIV clone sequencing revealed a new aminoacid sequence, at the N-terminus region, starting with a methionine residue.

By clone sequencing aminoacid sequences of GBV virus N-terminus were revealed. The "consensus" sequence is shown in Figure 4a.

Figur : 4b shows the reactivity obtained by assaying different sera with the peptide having the consensus sequence of Fig. 4a.

Peptide synthesis

Peptides were synthesized with an automated synthesizer MilliGen 9050 (Septrim) as shown for peptide for NS3 region.

Cases

sera from 20 healthy donors;
sera from 23 acute nonAnonE hepatitis patients;
sera from 10 chronic nonAnonE hepatitis patients;
sera from 22 acute hepatitis B patients;
sera from 33 drug addicted HIV-, HCV+ patients;
sera from 7 HIV+ patients;
sera from 17 HIV+ piastrinopenic patients;
sera from 47 Community Aquired HCV+ patients;
sera from 11 AIDS patients.

ELISA assay

Solid phase was prepared on polivinylchloride dishes (Nunc). 100 µl of a 20 µg/ml solution of G11C, D23C-VA, M37C-LVA, D13G-TG and M27C-CTG peptides, in carbonate buffer 50 mM, pH 9.6 were added to each well and incubated for 2 hrs at 37°C.

Further to peptide adsorbtion solid phases were saturated for 1 hr at 37°C with 300 µl/well of a BSA 2% solution in Tris-HCl 0.1M pH 7.5.

100 µl of 1:21 diluted serum in PBS containing 0.3 g/l EDTA, 2 g/l BSA, 2 ml/l Triton x100 and 2 ml/l Tween 20 were incubated into wells for 2 hrs at 37°C.

Following to 4 washes with PBS/Tween 20 0.1%, 100 µl/well of horseradish peroxidase conjugated goat anti human Ig (Sorin Biomedica), diluted 1:100 in a solution of PBS containing 0.04% Tween 20 and 20% of preimmune goat serum, were added to each well.

Following to 4 washes of PBS/Tween 20 0.1% BSA, 100 µl of chromogen and substrate were added to each well

(Sorin DEIA KIT). The colorimetric reaction was blocked after 30 min with 200 µl/well of sulphuric acid 1N. The OD reading was performed at 450 nm by referrng to OD at 630 nm.

Results show the presence of antibodies against peptides of the "core" region of GBV virus.

Sequence varialbility of the 5' terminal region of different GBV-C isolates

The GBV-C isolates so far analyzed have revealed putative truncated core proteins of about 83 and 46 amino acids in length (Linnen et al. supra, Simons et al. 1995 supra). These differences are due to nucleotide deletions or insertions that introduce in the sequences a frameshift causing the generation of distinct in-frame starting codons. It is conceivable, therefore, that the intrinsic genomic variability of this virus may introduce, in some particular isolates, additional mutations, insertions or deletions that could generate potentially functional core proteins.

In order to evaluate this possibility, we analyzed the nucleotide sequences, and their deduced translation products. On the basis of this analysis, we found 4 different types of sequences. The sequences of isolates 19, DM, DS39, BZ, 1159 and 47 encoded for a putative core protein of approximately 83 aminoacids in length, corresponding to the published sequence of R10291 isolate (GenBank accession no. U45966) (Linnen et al. supra) (GBV2 in Fig. 5 and 6). The most 5' and unique inframe AUG codon of isolates 3064 and MG, on the other hand, could initiate the translation of a putative core protein of 46 aminoacids in length similar to the one potentially encoded by the published sequences of GBV-C isolate (GenBank accession no. U36380) (Leary et al. supra) and PNF2161 isolate (Linnen et al. supra) (GenBank accession no. U44402) (GBV-C and GBV1 in the Fig. 5 and 6). The sequence of isolate 2095 was more peculiar since, due to

one base deletion (Fig. 5), it contained only one potential in-frame initiator AUG codon, downstream of the putative start codon of GBV-C and HGVI and located 9 nucleotides only upstream of the sequence that specifies the putative E1 signal sequence (Leary ET al. supra). The mostly unexpected finding, however, was that the sequence derived from the isolate 35 (35/1 in Fig. 5 and 6) could potentially encode for a putative core protein of approximately 106 aminoacids in length. This particular feature was due to a point mutation that introduces an additional in-frame AUG codon 69 nucleotides upstream of the putative initiation codon of the HGV 2 reference sequence.

In order to exclude the possibility of errors (intrinsic to the polymerase chain reactions) or of sequence misinterpretations (possibly due to the frequent sequence compressions), we confirmed our results by amplifying and sequencing several clones of each isolate (data not shown). Moreover, in the case of patient 35, we cloned and sequenced RT-PCR products derived from 2 additional sequential serum samples collected during the one year follow-up of this patient (sequences 35/2 and 35/3 in Fig. 6). While this analysis confirmed the presence of the upstream AUG codon in all the samples of patient 35, it also established that this particular isolate was stable over the time and that it was not dominantly replaced by a population of mutants.

Thus, our data provide the first evidence for the existence of additional GBV-C sequences, potentially leading to a core protein longer and not described in the previously cDNA clones derived from the numerous viremic patients analyzed so far (Leary et al., Simons et al. 1995, 1996, supra).

Finally, our results establish that the putative core proteins of our isolates are characterized by

discrete regions of high variability, that are separated by highly conserved motifs (Fig. 6).

Translation of isolates with distinct putative initiator codons

The deduced translation products derived from the nucleotide sequences of our isolates demonstrate that these GBV-C sequences are very heterogeneous with respect to the potential translation initiation site. Thus, in order to understand the potential replicative competence of these viral isolates, it was important to assess whether initiation at any of these sites can indeed occurr. This issue is particularly relevant since, recently, Simons et al. (1996 supra) have provided evidence suggesting that, in their GBV-C isolates, initiation of translation occurred only immediately upstream of the putative E1 signal sequence and that the upstream in frame AUG seemed to be unable to direct the synthesis of GBV-C polyprotein.

To determine the effect of the upstream in frame AUG codons on translation of our viral isolates, mRNAs with capped methylated (m^7GpppG) 5' end, synthesized from linearized pCRX/AC1S constructs, were examined for mRNA activity in a cell-free translation system prepared from rabbit reticulocytes. For this purpose we considered isolates with distinct putative initiator codons.

Isolate 35 had 5 in-frame AUG codons in the putative core region (Fig. 5 and 6) and therefore the pCR35/AC1S construct (covering the partial 5'UTR, the putative core and a portion of the E1 protein) can potentially mediate the synthesis of different transcription products with expected sizes of about 17, 14, 11, 10 or 7 kDa. Figure 7 shows that efficient translation occurred and a polypeptide with the apparent size of 17 kDa, compatible with the activity of the first in-frame AUG, was observed in a SDS-PAGE analysis. It is important to note that efficient translation was also obtained with IVTT

reactions programmed with uncapped RNA derived from pCR35/AC1S. Thus, these data strongly suggest that, at least in this construct, the first available in-frame AUG from the 5' end of the RNA can efficiently act as the initiator codon. Furthermore, since the cap stmcture was not determinant for efficient translation, the data also suggest that, as recently proposed by Simons et al. (Simons et al. 1996, supra), GBV-C RNA may not utilize the classical ribosome scanning mechanism.

IVTT reaction mixtures were also programmed with pCRDS39/AC1S, pCRDM/AC1S, pCR19/AC1S, pCR3064/AC1S, pCRMG/AC1S and pCR2095/AC1S constructs.

Isolates DS39, DM and 19 had the first in-frame AUG corresponding to the second in-frame AUG of isolate 35 (Fig. 5 and 6). Moreover, isolate DS39 had also two additional putative initiatior codons so that the pCRDS39/AC1S construct could potentially direct the synthesis of three possible proteins of 14, 10 and 7 kDa. The pCRDM/AC1S construct, on the other hand, can potentially initiate translation at 4 different AUG codons, producing polypeptides of 14, 11, 10 and 7 kDa.

Two of these plasmids, pCRDS39/AC1S and pCR19/AC1S, directed the translation of products of about 14 kDa as detected by SDS-PAGE (Fig. 7). The size of these proteins is compatible with the activity of the AUG sequence just downstream of the start codon of the pCR35/AC1S construct, suggesting that, also in these cases, translation was initiated at the first available in-frame AUG of the ORF. Since pCR19/AC1S contained only one AUG inframe with the long ORF, and since on the basis of the nucleotide sequence we could also exclude the possibility that the expressed proteins represented unrelated products of translation directed by AUG codon located in the other two frames, we conclude that the observed band is the correct product of translation.

In contrast, no bands were detected in the reactions programmed with the pCRDM/AC1S plasmid. Since this construct contained the same potential in-frame initiator AUG codon than pCR39/AC1S and pCR19/AC1S, the results rise the possibility that sequences upstream of the potential initiator codon may influence the translation either positively or negatively. This possibility is also reinforced by the fact that the plasmids derived from isolates 3064 and MG (pCR3064/AC1S and pCRMG/AC1S), that could potentially direct the synthesis of a band of 10 kDa, did not produce detectable quantities of specific proteins, in accordance to the data of Simons et al. (Simons et al. 1996 supra). Negative results were also obtained with the pCR2095/AC1S construct, derived from isolate 2095, that could potentially produce a protein of about 7 kDa.

Analysis of deletion mutants

To examine in details the activity of the different potential in-frame initiator AUG codons, to verify the possibility of a regulatory role of the upstream sequences and to unequivocally establish the size of the obtained translation products, cell-free protein synthesis experiments were performed with capped mRNA transcribed by truncated forms of the 35, 19, 3064 and 2095 constructs described in Fig. 8. Figure 9A shows the results obtained when IVTT reaction mixtures were programmed with the 35 deletion mutant plasmids. In these experiments, translation of the mRNA derived from pCR35/AC1S yielded the major precursor band of 17 kDa as well as 3 additional faint bands of 14, 11 and 7 kDa, probably corresponding to translation products that utilize the second, third and the fifth in-frame AUG as initiator codons. A very similar pattern was obtained with the pCR35/Δ1 construct. In vitro translation of pCR35/Δ2, pCR35/Δ3, pCR35/Δ4 and pCR35/Δ5 transcripts

yielded bands of progressively lower molecular weights of about 14, 11 and 7 kDa, implying therefore that, in this isolate, all the in-frame AUG codons, with the exception of the fourth AUG, might serve as potential site of translation initiation. In the lane containing the translation products of pCR35/Δ2 mutant we found two bands of 14 and 11 kDa corresponding to translation products that utilized both the second and the third available AUG initiation codons.

As expected, the analysis of the deletion mutants of pCR19/AC1S, that contains only one in-frame AUG, gave different results (Fig. 9B). The 14 kDa bands obtained with reactions programmed with pCR19/AC1S, pCR19/Δ1 and pCR19/Δ2 demonstrate that the first and unique in-frame AUG is the site of translation initiation and that, at least for this mutant, deletion of 5' UTR does not seem to significantly influence the level of translation.

A quite unexpected finding of these experiments was that the reactions programmed with some of the deletion mutants of pCR19/AC1S construct, that lacked the AUG codon immediately upstream of the putative E1 signal sequence, yielded a band with the same apparent molecular mass of the 7 kDa protein detected in the reactions programmed with pCR35/Δ4 and pCR35/Δ5. Although we can not exclude that this band may have resulted from partially degraded RNA, it is interesting that in this template the AUG triplet corresponding to the fifth in-frame start codon of isolate 35 is replaced by an ACG codon, that has been found to act as initiation codon in other mRNAs (Boeck, R., and D. Kolakofsky. 1994. EMBO J. 13:3608-3617), including a transcript derived from a GBV-C mutated sequence (Simons et al. 1996 supra).

Reactions programmed with the deletion mutants derived from pCR3064/AC1S construct provided no detectable bands (data not shown). Since in this

constructs the only available initiation codon is the AUG corrisponding to the fourth start codon of the isolate 35, these results confirmed the data obtained with the mutants of isolate 35 and with the pCR3064/AC1S and pCRMG/AC1S plasmids, suggesting that this particular codon, as already reported (Simons et al. 1996 supra), is silent, probably due to the composition of the flanking sequences.

Finally, evidence for a regulatory role of the partial 5'UTR were obtained with the deleted 2095 plasmids (Fig. 9C). In this isolate the only available site of transcription initiation corresponds to the fifth AUG of isolate 35, that is however silent in construct pCR2095/AC1S. The expected 7 kDa product was surprisingly detected as a faint band with pCR2095/Δ1, pCR2095/Δ2 and pCR2095/Δ3 and the intensity of this band increased progressively by programming the reactions with pCR2095/Δ4 and pCR2095/Δ5. These data clearly mean that, in this particular template, the translation of the polypeptide is strongly influenced by the partial 5'UTR present in the undeleted construct that, in this case, seems to inhibit the translation process.

Finally, we must stress that we have verified that none of the bands obtained with the deletion mutants can be accounted for by unrelated products of translation encoded by the other two possible reading frames.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Sorin Biomedica Diagnostics S.p.A.
(B) STREET: Via Borgonuovo 14
(C) CITY: Milan
(E) COUNTRY: ITALY
(F) POSTAL CODE (ZIP): 20121

(ii) TITLE OF INVENTION: NonA nonE hepatitis virus having a translatable core region, reagents and methods for their use

(iii) NUMBER OF SEQUENCES: 11

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 205 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Arg Val Arg Asp Val Ala Arg Gly Cys Gly Val Gln Leu Val Leu Tyr
1 5 10 15

Ala Thr Ala Thr Pro Pro Gly Ser Pro Met Thr Gln His Pro Ser Ile
20 25 30

Ile Glu Thr Lys Leu Asp Val Gly Glu Ile Pro Phe Tyr Gly His Gly

35 40 45

Ile Pro Leu Glu Arg Met Arg Thr Gly Arg His Leu Val Phe Cys His
50 55 60

Ser Lys Ala Glu Cys Glu Arg Leu Ala Gly Gln Phe Ser Ala Arg Gly
65 70 75 80

Val Asn Ala Ile Ala Tyr Tyr Arg Gly Lys Asp Ser Ser Ile Ile Lys
85 90 95

Asp Gly Asp Leu Val Val Cys Ala Thr Asp Ala Leu Ser Thr Gly Tyr
100 105 110

Thr Gly Asn Phe Asp Ser Val Thr Asp Cys Gly Leu Val Val Glu Glu
115 120 125

Val Val Glu Val Thr Leu Asp Pro Thr Ile Thr Ile Ser Leu Arg Thr
130 135 140

Val Pro Ala Ser Ala Glu Leu Ser Met Gln Arg Arg Gly Arg Thr Gly
145 150 155 160

Arg Gly Arg Ser Gly Arg Tyr Tyr Tyr Ala Gly Val Gly Lys Ala Pro
165 170 175

Ala Gly Val Val Arg Ser Gly Pro Val Trp Ser Ala Val Glu Ala Gly
180 185 190

Val Thr Trp Tyr Gly Met Glu Pro Asp Leu Thr Ala Asn
195 200 205

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

35 40 45

Arg Gly Ser Pro Arg Ile Leu Arg Val Arg Ala Gly Gly Ile Ser Leu
50 55 60

Pro Tyr Thr Ile Met Glu Ala Leu Leu Phe Leu Leu Gly Val Glu Ala
65 70 75 80

Gly Ala Ile Leu

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met Ser Leu Asn Arg Ala Arg Tyr Pro Pro Gly Leu Thr Thr Pro Thr
1 5 10 15

Tyr Gly Pro Arg Arg Pro Ser
20

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AGGGTTSGWW GGTSGTAAAT CC 22

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Ser Leu Asn Arg Ala Arg Tyr Pro Pro Gly Leu Thr Thr Pro Thr
1 5 10 15

Tyr Gly Pro Arg Arg Pro Ser Met Ser Leu Leu Thr Asn Arg Phe Asn
20 25 30

Arg Arg Val Asp Lys Asp Gln Trp Gly Pro Gly Val Met Gly Lys Asp
35 40 45

Pro Lys Pro Cys Pro Ser Arg Arg Thr Gly Lys Cys Met Gly Pro Pro
50 55 60

Ser Ser Ala Ala Ala Cys Ser Arg Gly Ser Pro Arg Ile Leu Arg Val
65 70 75 80

Arg Ala Gly Gly Ile Ser Leu Pro Tyr Thr Ile Met Glu Ala Leu Leu
85 90 95

Phe Leu Leu Gly Val Glu Ala Gly Ala Ile Leu
100 105

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 84 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Met Ser Leu Leu Thr Asn Arg Phe Asn Arg Arg Val Asp Lys Asp Gln
1 5 10 15

Trp Gly Pro Gly Val Met Gly Lys Asp Pro Lys Pro Cys Pro Ser Arg
20 25 30

Arg Thr Gly Lys Cys Met Gly Pro Pro Ser Ser Ala Ala Ala Cys Ser

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CGCCTGRTAN ARNGGCCARC A 21

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

ACGTAAGCTT AGGTGTWGGY CCTACCG 27

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

ACGTAAGCTT GTACGGYCCA CGTCGCC 27

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

ACGTAAGCTT GCGAGTTGRC AAGGACCAG

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

ACGTAAGCTT GRRVCGGGAA ATGCATG

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

ACGTAACCTT TGAGGGCGGG TGGCATT

CLAIMS

1. Peptide able to specifically bind to nonAnonE hepatitis virus antibodies, having a sequence of at least 10 aminoacids comprised either in SEQ ID No. 1:

RVRDVARGCGVQLVLYATATPPGSPMTQHPSIIETKLDVGEIPFYGHGIP
LERMRTGRHLVFCHSKAECERLAGQFSARGVNAIAYYRGKDSSIIKDGDL
VVCATDALSTGYTGNFDSVTDCGLVVEEVVEVTLDPTITISLRTVPASAE
LSMQRRGRTGRGRSGRYYYAGVGKAPAGVVRSGPVWSAVEAGVTWYGMEP
DLTAN;

or in SEQ ID No. 2:

MSLNRARYPPGLTTPTYGPRRPSMSLLTNRFNRRVDKDQWGPGVMGKDPK
PCPSRRTGKCMGPPSSAAACSRGSPRILRVRAGGISLPYTIMEALLFLLG
VEAGAIL;

or an immunologically homologous variant thereof.

2. Peptide able to specifically bind to nonAnonE hepatitis virus antibodies, having a sequence of at least 10 aminoacids, said nonAnonE hepatitis virus being characterized by having a translatable functional core-like protein; or an immunologically homologous variant thereof.

3. Peptide able to specifically bind to nonAnonE hepatitis virus antibodies according to claim 2 wherein said translatable functional core-like protein is longer than 84 aminoacid residues and comprises an aminoacid sequence at least 60% homologous to the sequence of SEQ ID No. 3: MSLLTNRFNRRVDKDQWGPGVMGKDPKPCPSRRTGKCMGPPSSAAA CSRGSPRILRVRAGGISLPYTIMEALLFLLGVEAGAIL;

or an immunologically homologous variant thereof.

4. Peptide able to specifically bind to nonAnonE hepatitis virus antibodies according to claim 2 wherein said translatable functional core-like protein longer than 84 aminoacid residues comprises an aminoacid sequence at least 60% homologous to the sequence of SEQ ID No. 4: MSLNRARYPPGLTTPTYGPRRPS;

or an immunologically homologous variant thereof.

5. Composition comprising at least one of the peptides according to any of previous claims.

6. Composition according to claim 5 which is adhered on a solid phase.

7. Composition according to claim 5 or 6 comprising at least two peptides according to any of claim 1-4.

8. Diagnostic kit for nonAnonE hepatitis comprising as specific reagent at least one peptide according to any of claim 1-4.

9. Diagnostic kit for nonAnonE hepatitis comprising as specific reagent at least two peptides according to any of claim 1-4.

10. Method to detect nonA nonE hepatitis virus antibodies in a sample comprising the following steps:

- incubating in conditions allowing the formation of an antigen-antibody complex said sample with at least one peptide according to any of claim 1-4;
- revealing said complex.

NS3 - PEPTIDES

1346

RVRDVARGCGVQLVLYATATPPGSPMTQHPSIIETKLDVGEIPFYGHGIPLERMRTGRHLVFCHSKAECERLAGQFSARGVNAIAYYRGKDSSIIKDGDLVVC 1448

R23G 1346-1368

S16V 1369-1384

S11Q 1410-1420

A19C 1430-1448

1449

ATDALSTGYTGNFDSVTDCGLVVEEVVEVTLDPTITISLRTVPASAELSMQRRGRTGRGRSGRYYYAGVGKAPAGVVRSGPVWSAVEAGVTWYGMEPDLTAN 1550

G20T 1459-1478

E21A 1495-1515

p3.1 1470
p3.2 1480
p3.3 1490
p3.4 1500
p3.5 1510
p3.6 1520
p3.7 1530
p3.8 1540
p3.9 1550

FIG. 1

REACTIVITY OF THE INDICATED SERA WITH PEPTIDES COVERING POSITION 1451-1550 OF THE NS3 REGION

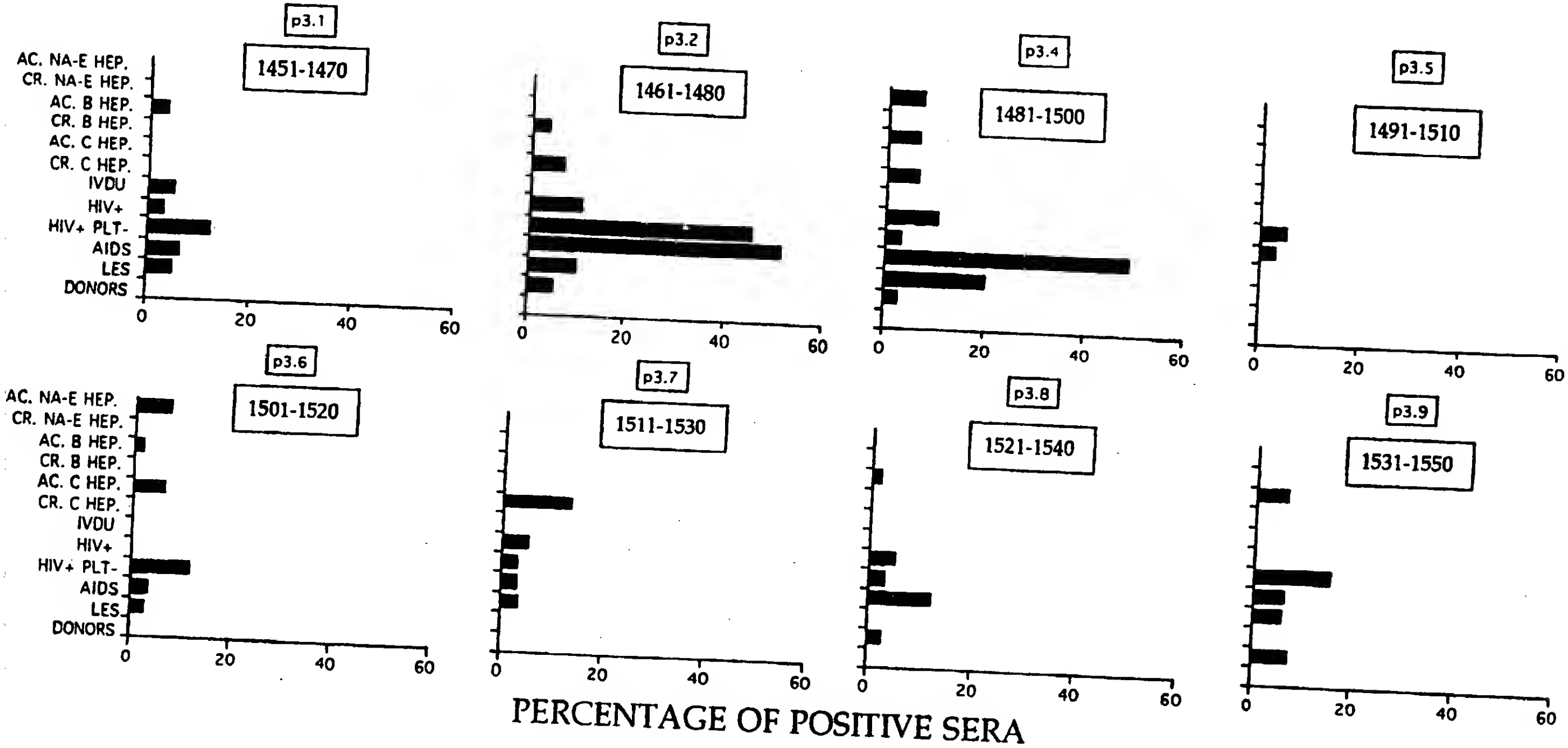


FIG. 2

WO 97/36929
PCT/IT96/00248
2/12

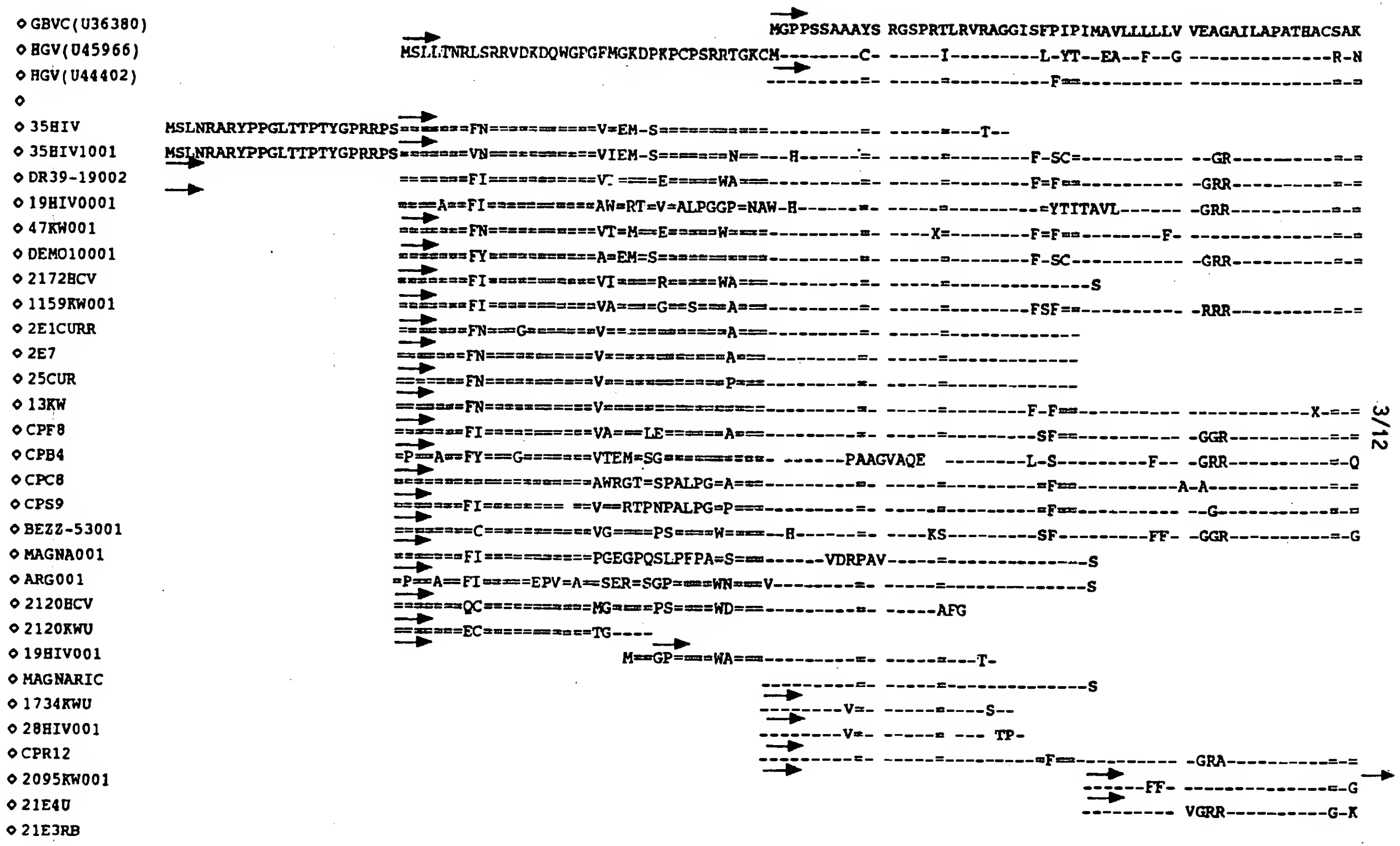

GBVC(U36380)
HGV(U45966)
HGV(U44402)
35HIV
35HIV1001
DR39-19002
19HIV0001
47KW001
DEMO10001
2172HCV
1159KW001
2E1CURR
2E7
25CUR
13KW
CPF8
CPB4
CPC8
CPS9
BEZZ-53001
MAGNA001
ARG001
2120HCV
2120KWU
19HIV001
MAGNARIC
1734KWU
28HIV001
CPR12
2095KW001
21E4U
21E3RB
MGPPSSAAAYS RGSPRTLRVRAGGISFPIPIMAVLLLLLV VEAGAILAPATHACSAK
MSLLTNRLSRRVDRDQWGFGFMGKDPRPCPSRRTGKCM
MSLNRARYPPGLTTPTYGPRRPS
MSLNRARYPPGLTTPTYGPRRPS

FIG. 3a

```
◇ GBVC4U36..     GQYXLTNCCALEDIGFCLEGGCLVALGCTICTDRCWPLYQAGLAVRPGKSAAQLVGELGSLYGPLSVSAYVAGILGLGEVYSGVLTVGVALTRRVYPVPNLTCAVECELKWESEFWR
◇ HGV(U45966)    ---F------P-----------------V-------------------------Q--G-------------------------------------------------
◇ HGV (U4402)    ---=------=---------------------Q-----------------------------------------------------M-------------------
◇                                                                                                      A----------
◇ 35HIV1001      ---=------=-----------------=----------
◇ DS39-10002     ---F------=-----------------------------
◇ 19HIV0001      ----------=-----------------------------
◇ DEMO10001      ---=------=-----------------=----------
◇ 47KW001        ---=------=-----------------V-------------------------------------------------------------------------------------
◇ 1159KW001      ---=------=-----------------V-----------F-------------------------------------------------=---------D----
◇ 13KW           ---=------=-----------------V-------------------------------------------------------------=---------E---------
◇ CPF8           ---=------=---------------                                                                 =-------------T----
◇ CPB4           ---=-----------------------------------
◇ CPC8           ---S------=-----------------------Q---F---
◇ CPS9           ---=------=--------------P---V-----------
◇ BEZZ-53001     ---=------=-----------------V---K---F---
◇ DS39-19001     ---F------=-----------------------------
◇ 2095KW001      ---=------=---E--------------V---K-----------------------------------------------------------------------------------
◇ 21E4U          ---F------M-----------------------------
◇ GBVC-UTR       GQYXLTNCCALEDIGFCLEGGCLVALGCTICTDRCWPLYQAGLAVRPGKSAAQLVGELGSLYGPLSVSAYVAGILGLGEVYSGVLTVGVALTRRVYPVPNLTCAVECELKWESEFWR
◇
◇
```

FIG. 3b

REACTIVITY OF THE INDICATED SERA WITH THE PEPTIDE DERIVED FROM THE CONSENSUS SEQUENCE OF THE CORE REGION

```
MSLLTNRLSRRVDKDQWGPGFMGKDPKPCPSRRTGK
 --------CW----G-----VR-RT-HAALPGGA-N
 -------FN----------V-EM-S----------
 -------VN----------VIEM-S-------RN-
 ----A--FI----------AW-RT-V-ALPGGP-N
 -------FN----------VT-M--E-----W---
 -------FY----------A-EM-S----------
 -------FI----------VI----R-----WA--
 -------FI----------VA----G--S---A--
 -------FN---G-------V------------A--
 -------FN----------V------------A--
 -------FN----------V------------P--
 -------FN----------V---------------
 -------FI----------VA---LE------A--
-P--A--FY---G-------VTEM-SG---------
 --------------------AWRGT-S-ALG-A---
 -------FI----------V--RTPN-ALPG-P--
 --------C----------VG----PS----W---
 -------FI----------PGEGPQSLPFPA-S--
-P--A--FI-----EPV-A--SER-SG-----WN--
 -------QC----------MG----PS----WD--
 -------EC----------TG----G-----WA--

MSLLTNRFNRRVDKDQWGPGVMGKDPKPSPSRRTGK   «CONSENSUS»
```

FIG. 4a

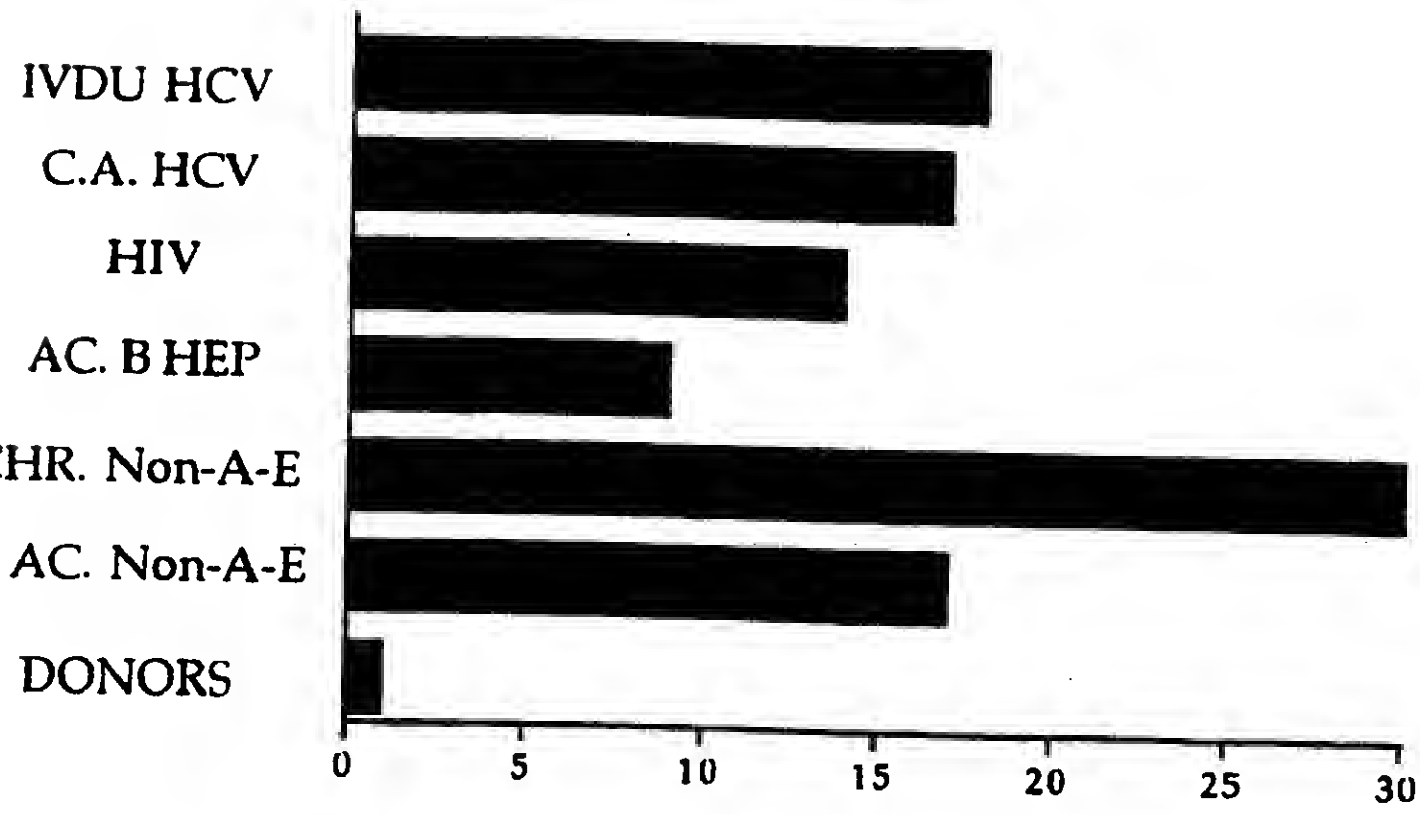


FIG. 4b

FIG. 5 (cont)

AC1S →

```
35/1    AGCGTTGGTAGGTCGTAAATCCCGGTCACCTTGGTAGCCACTATAGGTGGGTCTTAAGAGAAGGTTAAGATTCCTCTTGTGCCTGCGGCGAGACCGCGCA  -52
GBVC    AGCGTTGGTAGGTCGTAAATCCCGGTCATCCTGGTAGCCACTATAGGTGGGTCTTAAGGGGAGGCTACGGTCCCTCTTGCGCATATGGAGGAAAAGCGCA
HGV1    AGCGTTGGTAGGTCGTAAATCCCGGTCACCTTGGTAGCCACTATAGGTGGGTCTTAAGAGAAGGTTAAGATTCCTCTTGTGCCTGCGGCGAGACCGCGCA
HGV2    AGCGTTGGTAGGTCGTAAATCCCGGTCACCTTGGTAGCCACTATAGGTGGGTCTTAAGAGAAGGTTAAGATTCCTCTTGTGCCTGCGGCGAGACCGCGCA
19      AGCGTTGGATGGTGGTAAATCCCGGTCACCTTGGTAGCCACTATAGGTGGGTCTTAAGAGAAGGTTAAGATTCCTCTTGTGCCTGCGGCGAGACCGCGCA
DM      AGCGTTCGTAGGTCGTAAATCCCGGTCATCCTGGTAGCCACTATAGGTGGGTCTTAAGGGGAGGCTAAGATTCCTCTTGTGCCTGCCGCGAGACCGCGCA
DS39    AGCGTTCGTTGGTGGTAAATCCCGGTCATCCTGGTAGCCACTATAGGTGGGCCTTAAGAGAAGGTTAAGATTCCTCTTGTGCCTGCGGCGAGACCGCGCA
BZ      AGCGTTGGTCGGTCGTAAATCCCGGTCATCTTGGTAGCCACTATAGGTGGGTCTTAAGGGAAGGTTAAGATTCCTCTTGTGCCTGTGGCGAGACAGCGCA
1159    AGCGTTGGTAGGTGGTAAATCCCGGTCACCTTGGTAGCCACTATAGGTGGGTCTTAAGAGAAGGTTAAGATTCCTCTTGTGCCTGCGGCGAGACCGCGCA
47      AGCGTTGGTAGGTCGTAAATCCCGGTCATCCTGGTAGCCACTATAGGTGGGTCTTAAGAGAAGGTTAAGAATCCTCTTGTGCCTGCGGCGAGACCGCGCA
3064    AGCGTTCGTTGGTGGTAAATCCCGGTCACCTTGGTAGCCACTATAGGTGGGTCTTAAGAGAAGGTTAAGATTCCTCTTGTGCCTGCGGCGAGACCGCGCA
MG      AGCGTTGGAAGGTGGTAAATCCCGGTCACCTTGGTAGCCACTATAGGTGGGTCTTAAGAGAAGGTTAAGATTCCTCTTGTGCCTGCGGCGAGACCGCGCA
2095    AGCGTTGGAAGGTCGTAAATCCCGGTCATCTTGGTAGCCACTATAGGTGGGTCTTAAGGGGAGGCTAAGATTCCTCTTGTGCCTGTCGCGAGACAGCGCA
                                                                                                              6/12
```

Met

```
35/1    CGGTCCACAGGTGTAGGCCCTACCGGTGTGACTAAGGGCCCGACATCAGGCATGTCGTTAAACCGAGCCCGTTATCCGCCTGGGCTAACGACGCCCACGT  +49
GBVC    CGGTCCACAGGTGTTGGTCCTACCGGTGT-AATAAGGACCCGGCGCTAGGCACGCCGTTAAACCGAGCCCGTTACTCCCCTGGGCAAACGACGCCCACGT
HGV1    CGGTCCACAGGTGTTGGCCCTACCGGTGGGAATAAGGGCCCGACGTCAGGCTCGTCGTTAAACCGAGCCCGTTACCCACCTGGGCAAACGACGCCCACGT
HGV2    CGGTCCACAGGTGTTGGCCCTACCGGTGGGAATAAGGGCCCGACGTCAGGCTCGTCGTTAAACCGAGCCCGTAACCCGCCTGGGCAAACGACGCCCACGT
19      CGGTCCACAGGTGTTGGCCCTACCGGTGTGAATAAGGGCCCGACATCAGGCTTGTCGTTAAACCGAGCCCGTTACCCGCCTGGGCAAACGACGCCCACGT
DM      CGGTCCACAGGTGTTGGCCCTACCGGTGTGAATAAGGGCCCGACATCAGGCTTGTCGTTAAACCGAGCCCGTAATCCGCCTGGGCTAACGACGCCCACGT
DS39    CGGTCCACAGGTGTTGGCCCTACCGGTGTGAATAAGGGCCCGACATCAGGCTTGTCGTTAAACTGAGCCCGTTACCCACCTGGGCTAACGACGCCCACGT
BZ      CGGTCCACAGGTGTTGGCCCTACCGGTGTGAATAAAGGCCCGACGTCAGGCTCGTCGTTAAACCGAGACCGACACCCACCTGGGCAAACGACGCCCACGT
1159    CGGTCCACAGGTGTTGGCCCTACCGGTGTGAATGAGGGCCCGACGTCAGGCTCGTCGTTAAACTGAGCCCGTTACCCGCCTGGGCAAACGACGCCCACGT
47      CGGTCCACAGGTGTTGGCCCTACCGGTGTGAATAAGGGCCCGACATCAGGCTTGTCGTTAAACCGAGCCCGTAATCCACCTGGGCAAACGACGCCCACGT
3064    CGGTCCACAGGTGTTGGCCCTACCGGTGTGAATAAGGGCCCGACATCAGGCTTGTCGTTAAACCGAGCCCGTTACCCGCCTGGGCAAACGACGCCCACGT
MG      CGGTCCACAGGTGTTGGCCCTACCGGTGTGAATAAGGGCCCGACATCAGGCTTGTCGTTAAACCGAGCCCGTTACCCGCCTGGGCAAACGACGCCCACGT
2095    CGGTCCACAGGTGTTGGCCCTACCGGTGTGAATAAGGGCCCGACGTCAGGCTCGTCGTTAAACCGAGACCGACACCCACCTGGGCAAACGACGCCCACGT
```

FIG. 5(cont)

```
                                 Met                                                                                     Met
35/1   ACGGTCCACGTCGCCCTTCAATGTCTCTCTTGACCAATAGGGTTAACCGGCGAGTTGACAAGGACCAGTGGGGGCCGGGGTTATAGACATGGACTCCAA  +149
GBVC   ACGGTCCACGTCGCCCTTCAATGTCTCTCTTGACCAATAGGCGTA--CGGCGAGTTGACAAGGACCAGTGGGGGCCGGGCGGAGGGGGAAGGACCCCCA
HGV1   ACGGTCCACGTCGCCCTTCAATGTCTCTCTTGACCAATAGGCGTAGCCGGCGAGTTGACAAGGACCAGTGGGGGCCGGGGGC-TTGGAGAGGGACTCCAA
HGV2   ACGGTCCACGTCGCCCTTCAATGTCGCTCTTGACCAATAGGCTTAGCCGGCGAGTTGACAAGGACCAGTGGGGGCCGGGGTTTATGGGAAGGACCCCAA
19     ACGGTCCACGTCGCCCTTCAATGTCTCTCTTGGCCAATAGGTTTATCCGGCGAGTTGACAAGGACCAGTGGGGGCCGGGGGC-CTGGGAAGGACCCCAG
DM     ACGGCCCACGTCGCCCTTCAATGTCTCTCTTGACCAATAGGTTTACCGGCGAGTTGACAAGGACCAGTGGGGGCCGGGGGCTATGGACATGGACTCCAA
DS39   ACGGTCCACGTCGCCCTTCAATGTCTCTCTTGACCAATAGGTTTATCCGGCGAGTTGACAAGGACCAGTGGGGGCCGGGGGTTACGGGGAAGGACCCCGA
BZ     ACGGTCCACGTCGCCCTTCAATGTCTCTCTTGACCAATAGGCTTTGCCGGCGAGTTGACAAGGACCAGTGGGGGCCGGGGGTTGGAGGGAAGGACCCTCC
1159   ACGGTCCACGTCGCCCTTCAATGTCTCTCTTGACCAATAGGTTTATCCGGCGAGTTGACAAGGACCAGTGGGGGCCGGGGGTTGCGGGGAAGGACCCCGG
47     ACGGTCCACGTCGCCCTTCAATGTCTCTCTTGACCAATAGGTTTAACCGGCGAGTTGACAAGGACCAGTGGGGGCCGGGGGTTACGGGCATGGACCCCGA
3064   ACGGTCCACGTCGCCCTTCAATGTCTCTCTTGACCAATAGGTTTATCCGGCGAGTTGACAAGGACCAGTGGGGGCCGGGGGC-CTGGGGAAGGACCCCAG
MG     ACGGTCCACGTCGCCCTTCAATGTCTCTCTTGACCAATAGGTTTATCCGGCGAGTTGACAAGGACCAGTGGGGGCCGGGGGC-CTGGGAAGGACCCCAG
2095   ACGGTCCACGTCGCCCTTCAATGTCTCTCTTGACCAATAGGCAATGCCGGCGAGTTGACAAGGACCAGTGGGGGCCGGGGACGGGGGGGAAGGACCCCCC
       **** ****************** ****** ********     ****************************** ****        * ** **** *

                                      Met
35/1   ACCCTGCCCTTCCCGGCGGAACGGGAAATGCATGGGC-ACCCCAGCTCCGCGGCGGCCTGCAGCCGGGGTAGCCCAAGAATCCTTCGGGTGAGGGCGGG  +248
GBVC   CCGCTGCCCTTCCCGGGGAGGCGGGAAATGCATGGGCCACCC-AGCTCCGCGGCGGCCTACAGCCGGGGTAGCCCAAGAACCCTTCGGGTGAGGGCGGG
HGV1   GTCCCGCCCTTCCCGGTGGGCCGGGAAATGCATGGGCCACCC-AGCTCCGCGGCGGCCTGCAGCCGGGGTAGCCCAAGAATCCTTCGGGTGAGGGCGGG
HGV2   ACCCTGCCCTTCCCGGCGGACCGGGAAATGCATGGGCCACCC-AGCTCCGCGGCGGCCTGCAGCCGGGGTAGCCCAAGAATCCTTCGGGTGAGGGCGGG
19     TCCCTGCCCTTCCCGGCGGGCCGGGAAATGCATGGGGCCACCCCAGCTCCGCGGCGGCCTGCAGCCGGGGTAGCCCAAGAATCCTTCGGGTGAGGGCGGG
DM     GCCCTGCCCTTCCCGGCGGACCGGGAAATGCATGGGCCACCC-AGCTCCGCGGCGGCCTGCAGCCGGGGTAGCCCAAGAATCCTTCGGGTGAGGGCGGG
DS39   ACCCTGCCCCTCCCGGTGGGCCGGGAAATGCATGGGCCACCC-AGCTCCGCGGCGGCCTGCAGCCGGGGTAGCCCAAGAATCCTTCGGGTGAGGGCGGG
BZ     ATCCTGCCCTTCCCGGTGGACCGGGAAATGCATGGG-CCACCCCAGCTCCGCGGCGGCCTGCAGCCGGGGTAGCCCAAAGAGCCTTCGGGTGAGGGCGGG
1159   ACCCTGCTCTTCCCGGCGGGCCGGGAAATGCATGGGCCACCC-AGCTCCGCGGCGGCCTGCAGCCGGGGTAGCCCAAGAATCCTTCGGGTGAGGGCGGG
47     ACCCTGCCCTTCCCGGTGGACCGGGAAATGCATGGGCCACCC-AGCTCCGCGGCGGCCTGCAGCCGGGGTAGCCCAAGAATCCTTCGGGTGAGGGCGGG
3064   TCCCTGCCCTTCCCGGCGGGCCGGGAAATGCATGGGCCACCC-AGCTCCGCGGCGGCCTGCAGCCGGGGTAGCCCAAGAATCCTTCGGGTGAGGGCGGG
MG     TCCCTGCCCTTCCCGGCGGGCCGGGAAATGCATGGGCCACCC-AGCTCCGCGGCGGCCTGCAGCCGGGGTAGCCCAAGAATCCTTCGGGTGAGGGCGGG
2095   CTCCTGCCCATCCCGGTGGGACGGGAAATGCATGGGGCCACCC-AGCTCCGCGGCGGCCTGCAGCCGGGGTAGCCCAA-GAGCCTTCGGGTGAGGGCGGG
          * ** * ****** *     ************** * **** *************** *************** * ***************
```

FIG. 5

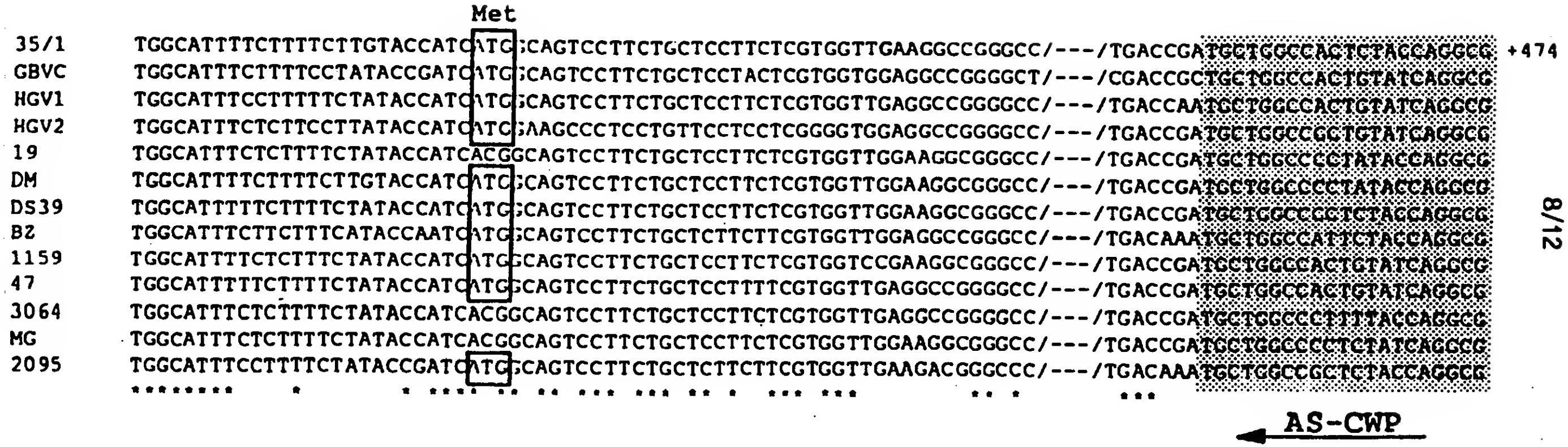
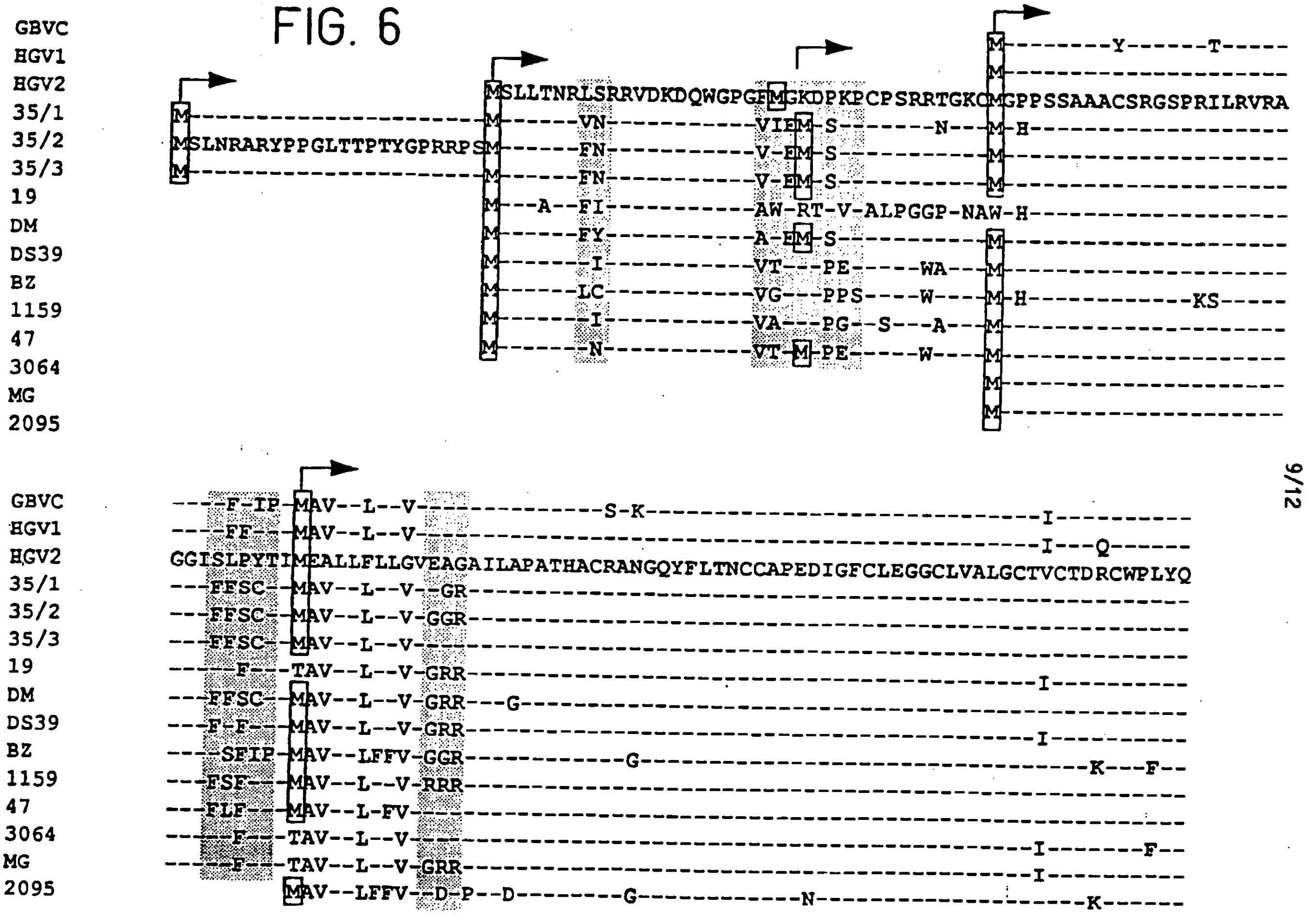

Met
35/1 TGGCATTTTCTTTTCTTGTACCATCATGCAGTCCTTCTGCTCCTTCTCGTGGTTGAAGGCCGGGCC/---/TGACCGATGCTGGCCACTCTACCAGGCG +474
GBVC TGGCATTTCTTTTCCTATACCGATCATGCAGTCCTTCTGCTCCTACTCGTGGTGGAGGCCGGGGCT/---/CGACCGCTGCTGGCCACTGTATCAGGCG
HGV1 TGGCATTTCCTTTTTCTATACCATCATGCAGTCCTTCTGCTCCTTCTCGTGGTTGAGGCCGGGGCC/---/TGACCAATGCTGGCCACTGTATCAGGCG
HGV2 TGGCATTTCTCTTCCTTATACCATCATGCAAGCCCTCCTGTTCCTCCTCGGGGTGGAGGCCGGGGCC/---/TGACCGATGCTGGCCGCTGTATCAGGCG
19 TGGCATTTCTCTTTTCTATACCATCACGGCAGTCCTTCTGCTCCTTCTCGTGGTTGGAAGGCGGGCC/---/TGACCGATGCTGGCCCCTATACCAGGCG
DM TGGCATTTTCTTTTCTTGTACCATCATGCAGTCCTTCTGCTCCTTCTCGTGGTTGGAAGGCGGGCC/---/TGACCGATGCTGGCCCCTATACCAGGCG
DS39 TGGCATTTTTCTTTTCTATACCATCATGCAGTCCTTCTGCTCCTTCTCGTGGTTGGAAGGCGGGCC/---/TGACCGATGCTGGCCGCTCTACCAGGCG
BZ TGGCATTTCTTCTTTCATACCAATCATGCAGTCCTTCTGCTCTTCTTCGTGGTTGGAGGCCGGGCC/---/TGACAAATGCTGGCCATTCTACCAGGCG
1159 TGGCATTTTCTCTTTCTATACCATCATGCAGTCCTTCTGCTCCTTCTCGTGGTCCGAAGGCGGGCC/---/TGACCGATGCTGGCCACTGTATCAGGCG
47 TGGCATTTTTCTTTTCTATACCATCATGCAGTCCTTCTGCTCCTTTTCGTGGTTGAGGCCGGGGCC/---/TGACCGATGCTGGCCACTGTATCAGGCG
3064 TGGCATTTCTCTTTTCTATACCATCACGGCAGTCCTTCTGCTCCTTCTCGTGGTTGAGGCCGGGGCC/---/TGACCGATGCTGGCCCCTTTTACCAGGCG
MG TGGCATTTCTCTTTTCTATACCATCACGGCAGTCCTTCTGCTCCTTCTCGTGGTTGGAAGGCGGGCC/---/TGACCGATGCTGGCCCCTCTATCAGCCG
2095 TGGCATTTCCTTTTCTATACCGATCATGCAGTCCTTCTGCTCTTCTTCGTGGTTGAAGACGGGCCC/---/TGACAAATGCTGGCCGCTCTACCAGGCG
AS-CWP

FIG. 6



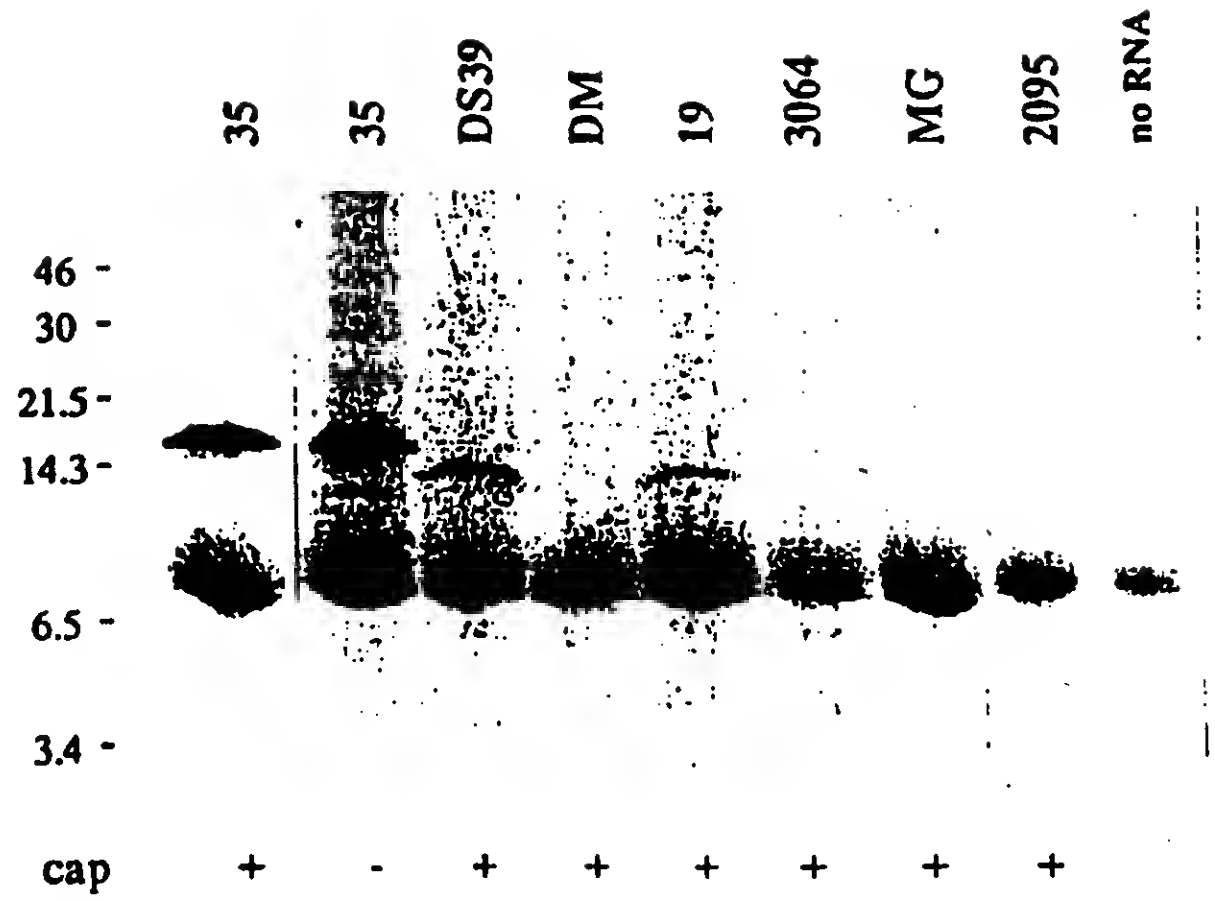


FIG. 7

FIG. 8

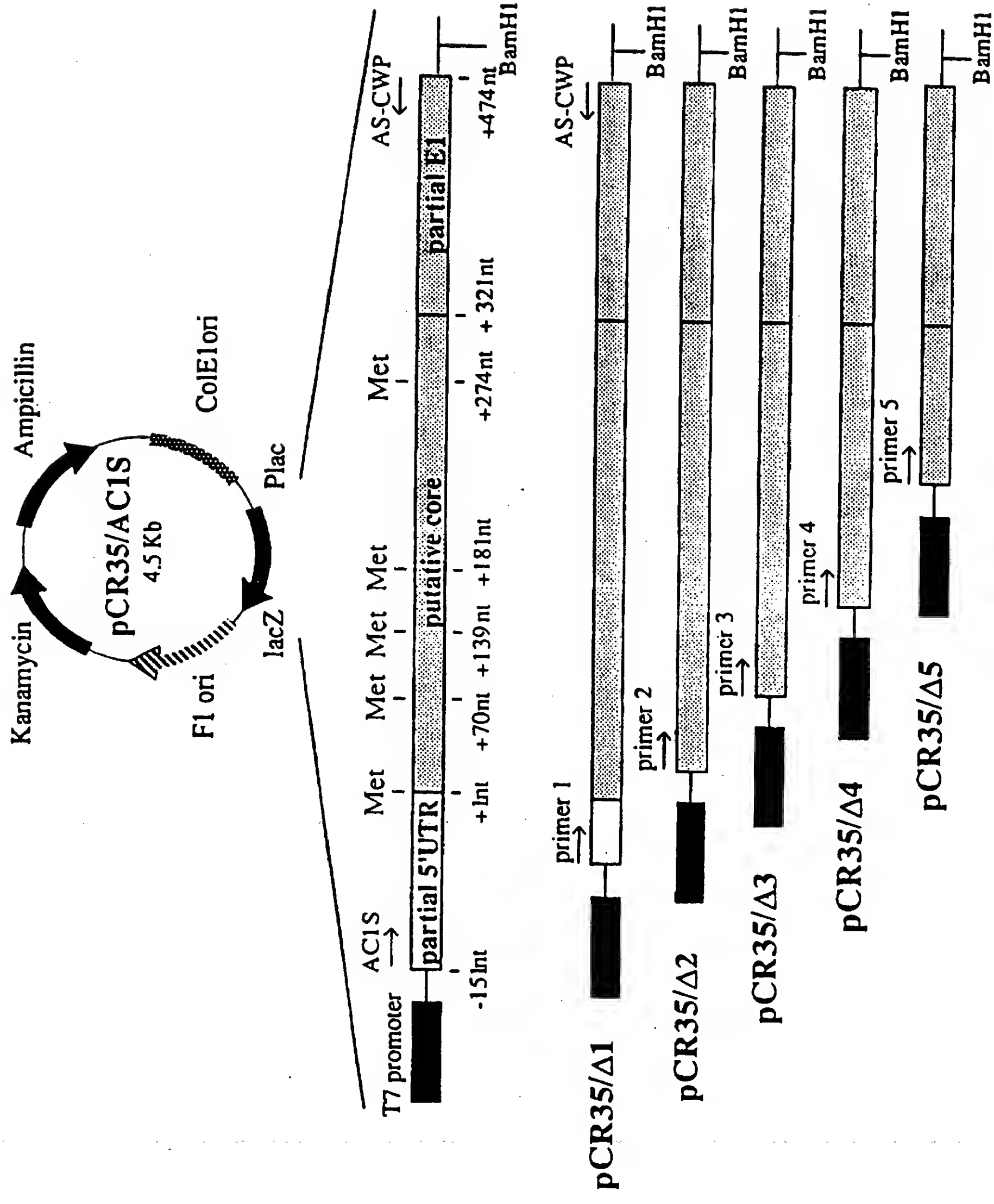
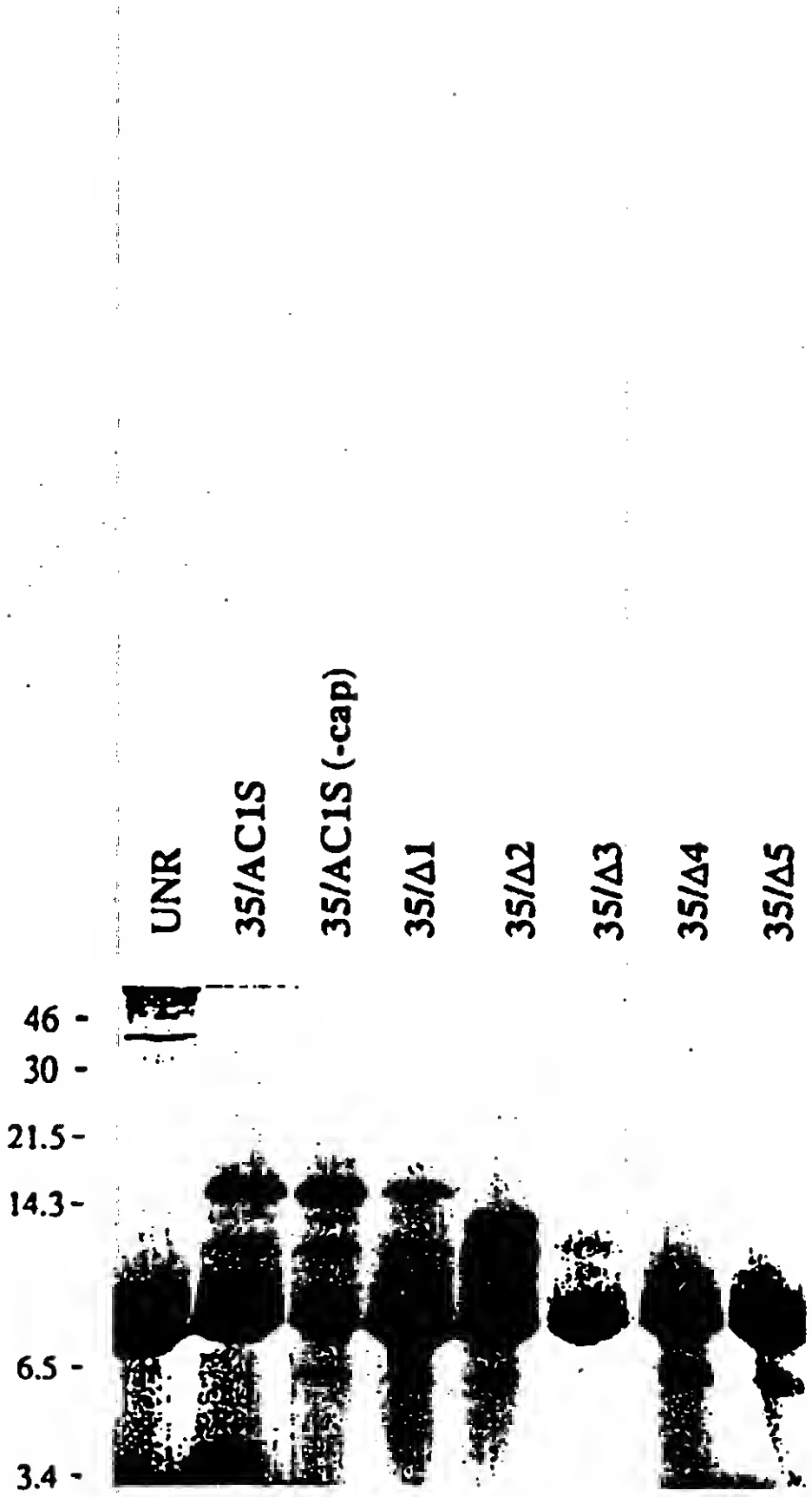
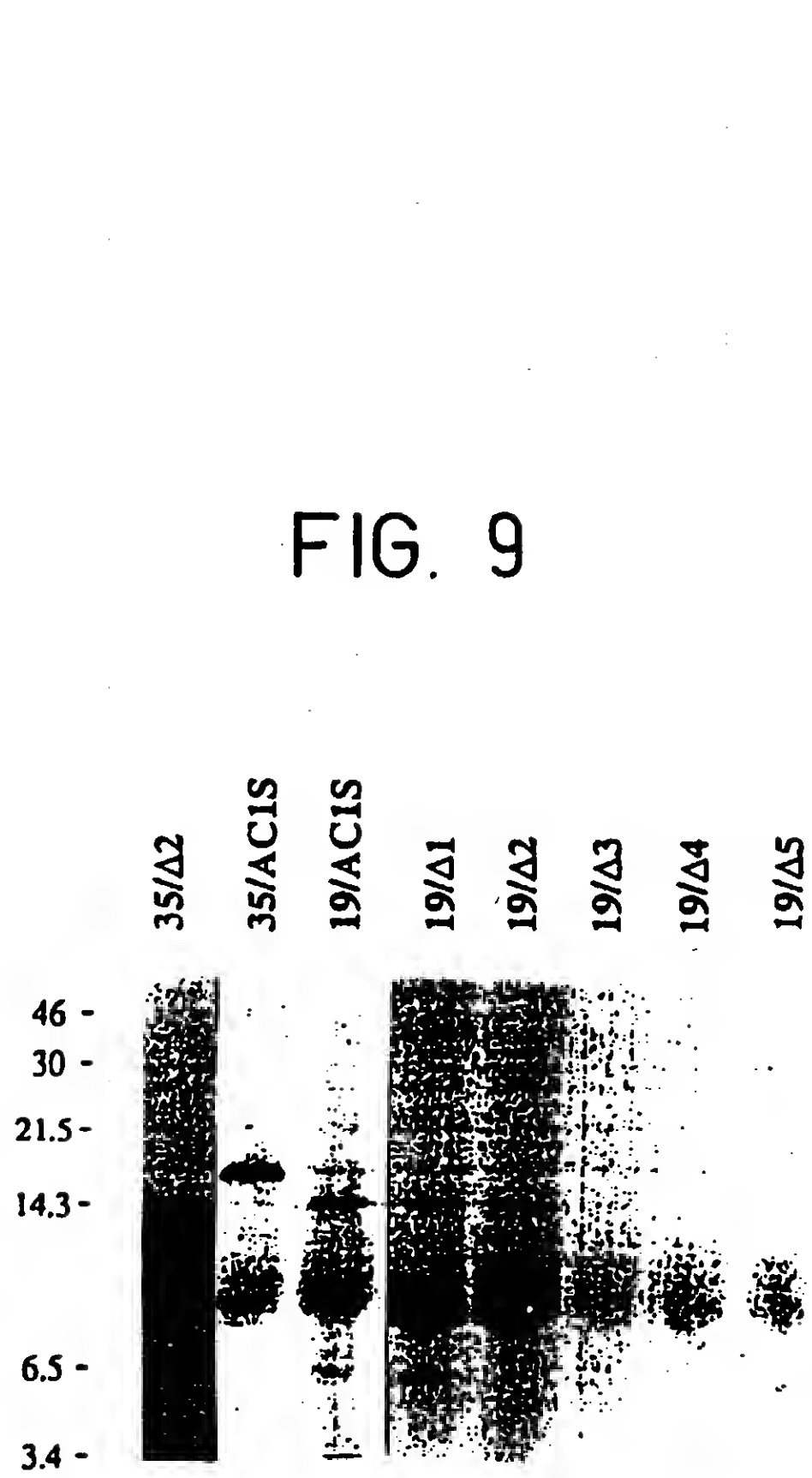

pCR35/AC1S
4.5 Kb
Ampicillin
Kanamycin
ColE1ori
F1 ori
Plac
lacZ
AC1S
T7 promoter
partial 5'UTR
-15 1nt
+1nt
Met
putative core
partial
-70nt +139nt +181nt
+274nt +321nt
BamH1
+474 nt
primer 1
primer 2
primer 3
primer 4
primer 5
pCR35/Δ1
pCR35/Δ2
pCR35/Δ3
pCR35/Δ4
pCR35/Δ5

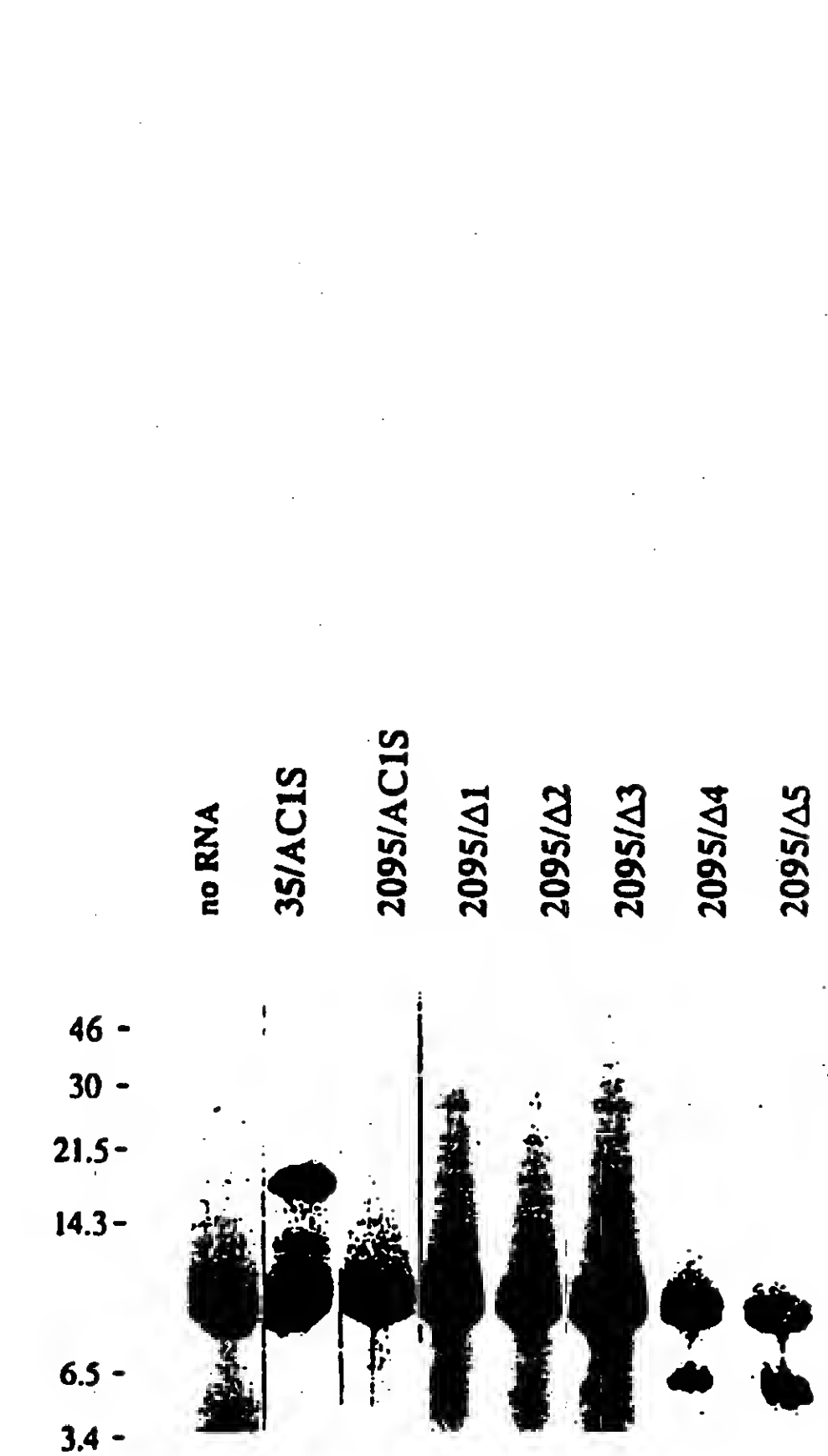
FIG. 9


UNR
35/AC1S
35/AC1S (-cap)
35/Δ1
35/Δ2
35/Δ3
35/Δ4
35/Δ5
46 -
30 -
21.5 -
14.3 -
6.5 -
3.4 -

(A)


35/Δ2
35/AC1S
19/AC1S
19/Δ1
19/Δ2
19/Δ3
19/Δ4
19/Δ5
46 -
30 -
21.5 -
14.3 -
6.5 -
3.4 -

(B)


no RNA
35/AC1S
2095/AC1S
2095/Δ1
2095/Δ2
2095/Δ3
2095/Δ4
2095/Δ5
46 -
30 -
21.5 -
14.3 -
6.5 -
3.4 -

(C)

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/IT 96/00248

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/51 C07K14/18 G01N33/576

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 32292 A (GENELABS TECH INC ;FRY KIRK E (US); KIM JUNGSUH P (US); MURPHY FRE) 30 November 1995 see the whole document ---	1-10
X	WO 95 32291 A (GENELABS TECH INC) 30 November 1995 cited in the application see the whole document ---	1-3,5-10
X	NATURE MEDICINE, vol. 1, no. 6, 1 June 1995, pages 564-569, XP000508526 SIMONS J N ET AL: "ISOLATION OF NOVEL VIRUS-LIKE LEQUENCES ASSOCIATED WITH HUMAN HEPATITIS" cited in the application see the whole document ---	1,2,5-10

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
8 April 1997	16. 05. 97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer Groenendijk, M

INTERNATIONAL SEARCH REPORT

Intern nal Application No
PCT/IT 96/00248

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SCIENCE, vol. 271, 26 January 1996, LANCASTER, PA US, pages 505-508, XP002028989 J.LINNEN E.A.: "Molecular cloning and disease association of Hepatitis G virus: a transfusion-transmissible agent" cited in the application see the whole document -----	1-10

INTERNATIONAL SEARCH REPORT

.nformation on patent family members

Interr ıal Application No
PCT/IT 96/00248

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		EP 0763114 A	19-03-97
		NO 964721 A	17-01-97
		WO 9532291 A	30-11-95
WO 9532291 A	30-11-95	AU 2594195 A	18-12-95
		AU 2689595 A	18-12-95
		CA 2190860 A	30-11-95
		EP 0763114 A	19-03-97
		NO 964721 A	17-01-97
		WO 9532292 A	30-11-95